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Effect of low-concentration rhamnolipid on transport of *Pseudomonas aeruginosa* ATCC 9027 in ideal porous medium with hydrophilic or hydrophobic surfaces

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Abstract

The success of effective bioaugmentation processes for remediation of soil and groundwater contamination requires effective transport of the injected microorganisms in the subsurface environment. In this study, the effect of low concentrations of monorhamnolipid biosurfactant solutions on transport of *Pseudomonas aeruginosa* in an ideal porous medium (glass beads) with hydrophilic or hydrophobic surfaces was investigated by conducting miscible-displacement experiments. Transport behavior was examined for both glucose-grown and hexadecane-grown cells, with low or high surface hydrophobicity, respectively. A clean-bed colloid deposition model was used for determination of deposition rate coefficients. Results show that cells with high surface hydrophobicity exhibit greater retention than cells with low surface hydrophobicity. Rhamnolipid affects cell transport primarily by changing cell surface hydrophobicity, with an additional minor effect by increasing solution ionic strength. There is a good linear relation between k rhamnolipid-regulated cell surface hydrophobicity presented as bacterial-adhesion-to-hydrocarbon (BATH) rate of cells ($R^2 = 0.71$). The results of this study show the importance of hydrophobic interaction for transport of bacterial cells in silica-based porous media, and the potential of using low-concentration rhamnolipid solutions for facilitating bacterial transport in bioaugmentation efforts.

Keywords

rhamnolipid; cell surface hydrophobicity; deposition; hydrophobic interaction; bacterial transport

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1 Introduction

Microorganisms are used for subsurface remediation to enhance the degradation or transformation of contaminants either by adding nutrients to stimulate the growth of indigenous subsurface populations (biostimulation), or by directly injecting bacterial consortia into the subsurface (bioaugmentation). Successful bioaugmentation relies on adequate distribution of the injected microorganisms throughout the contaminated site. However, it is well established that bacterial transport through geologic media such as soil and sediment is subject to significant retention, wherein the concentration of suspended bacteria is typically reduced by several orders of magnitude over transport distances of 10's to 100's of cm, resulting in failure of the bioaugmentation process. A growing research effort has thus been focused on developing methods for improving bacterial transport in geomedia [1–5].

The subsurface transport of microorganisms is typically treated as a colloid transport process, governed by advective-dispersive dynamics and deposition to the solid matrix. Deposition of bacterial cells in geomedia appears to be affected by (1) microbial properties including surface charge [6], hydrophobicity [7, 8], bacterial size and shape [9], growth phase [10], motility [11], nutrition condition [12], chemotaxis [13], metabolic activity [14], and composition of the cell surface (e.g., lipopolysaccharide, extracellular polymeric substances, outer membrane proteins, flagella and fimbriae) [15–19], (2) soil properties including soil particles size and surface properties [17, 20], mineral content [21], moisture content [7] and organic matter or nutrients content [22, 23], (3) environmental factors including ionic strength and ion valence [24, 25], pH [26] and temperature [27]. In addition, hydraulic conditions such as flow velocity [28, 29], input microbial concentration [30] and heterogeneity of microbial populations [6] also play a role.

Biosurfactants are surfactants produced by microorganisms. Biosurfactants have the potential to affect bacterial attachment and transport behavior in porous media by changing the electrostatic, hydrophobic, and steric interactions between cells and solids [e.g., 31]. Rhamnolipid is one of the most extensively studied biosurfactants and it shows the capability of affecting bacterial adsorption and transport in porous media. For example, adsorption of bacteria, e.g. *Alcaligenes paradoxus*, *Pseudomonas aeruginosa*, *Lactobacillus casei*, and *Streptococcus mitis*, on glass beads, silica powder, or soil, decreased in the presence of monorhamnolipid [2–4].

Most of these previous studies were conducted at relatively high rhamnolipid concentrations. High cost for rhamnolipid supply may limit their use at high concentrations for field applications. Recent studies show that rhamnolipid is effective in changing cell surface properties at relatively low concentrations (~10 mg/L) [32–34]. In our prior batch experiment study, monorhamnolipid with concentration as low as 20 mg/L was observed to effectively weaken hydrophobic interaction between *P. aeruginosa* cells and glass beads, and thus reduce cell adsorption [35]. Therefore, low-concentration rhamnolipid has the potential to enhance bacterial transport in porous media.

The objective of this study is to investigate the relation between bacterial cell surface hydrophobicity regulated by low-concentration rhamnolipid and cell transport in porous media. A Gram-negative bacterium, *Pseudomonas aeruginosa* ATCC 9027, was cultured with glucose or hexadecane and used as the source of cells. Miscible-displacement column experiments were conducted using glass beads with two types of surfaces, hydrophilic or hydrophobic. A clean-bed colloid deposition model was used to obtain deposition rate coefficients. In addition, the mechanisms governing the impact of rhamnolipid on bacterial transport behavior were explored by monitoring cell surface properties (hydrophobicity and zeta potential).

2 Materials and methods

2.1 Bacterium, porous media and chemicals

The strain of *P. aeruginosa* ATCC 9027 was obtained from the American Type Culture Collection (Rockville, Md.). It was maintained in lyophilized stock at -30°C and revived on peptone agar slant before use. Cells were grown in the presence of hexadecane to produce cells with hydrophobic surfaces, and in the presence of glucose to produce cells with hydrophilic surfaces.

Preparation of glass beads with hydrophilic or hydrophobic surfaces was as described in Zhong et al [35]. Morphology of the glass beads was examined by scanning electron microscope (Tabletop-SEM, TM3000, Hitachi, Japan) under vacuum. The glass beads are observed to be perfectly spherical with smooth surfaces, and a diameter of approximately 0.35 mm.

The monorhamnolipid (monoRL, 99%) was purchased from Huzhou Zijin Biotechnology Co., Ltd. (Zhejiang, China). The methods and results for component analysis are presented in ref. [35]. Hexadecane (purity 99%) was obtained from Sigma-Aldrich (St. Louis, MO). All the other chemicals are of analytical grade and used as received. The water used for the experiment is produced by UPT-II-40 (Ulupure, Chengdu, China) with electrical resistivity of $18.2\text{ m}\Omega\cdot\text{cm}$.

2.2 Miscible-displacement experiments

The columns used for the transport experiments were 15.4 cm in length and 2.2 cm in inner diameter. The body of the column was constructed of tempered glass. Dense wire-net frits were placed at both ends of the column to retain the porous medium and promote uniform flow. The frits and all connectors were constructed of stainless steel.

The glass beads were dry packed into the column under vibration. The packed columns were flushed with CO_2 to displace air and then saturated with de-aired sterile artificial ground water (AGW, ingredients per liter: 0.006 g NaCl, 0.012 g CaSO_4 , 0.012 g NaHCO_3 , 0.002 g KNO_3 , 0.035 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ [3]) using a valveless piston pump (QG-6, Fluid Metering INC, USA) to provide constant flow to the bottom of the vertically oriented column. The porosity of the glass beads column was determined gravimetrically and is approximately 0.33. Nonreactive tracer tests employing pentafluorobenzoic acid (PFBA, 80 mg/L) were

conducted before injection of bacterial suspensions to characterize the hydrodynamic properties of the packed columns.

The methods and procedures for cell growth and harvest, and for preparation of cell suspension in AGW with rhamnolipid, were described by Zhong et al. [35]. Before the bacterial suspension was used for injection into the column, it was allowed to stand for an hour to guarantee full interaction between rhamnolipid and cells [35]. Then the cell surface hydrophobicity was measured using the bacterial-adhesion-to-hydrocarbons (BATH) method [36]. Zeta potential of the cells was measured using a ZEN3600 Zetasizer (Malvern Instruments, Malvern, UK) as described by Zhong et al. [37].

Each miscible-displacement experiment was conducted as follows. The packed column was first equilibrated with AGW by flushing the column with ~30 pore volume equivalents (PVs) of AGW. Then 4 to 5 PVs of cell suspension were introduced into the bottom of the vertically oriented column at a constant flow rate of 0.30 mL/min, followed by 4 to 5 PVs of cell-free solution. The effluent from the column was collected every 15 min in 10-mL tubes using a BSZ-100 automatic fraction collector (Shanghai, China). The optical density of samples at 600 nm (OD_{600}) was determined with a Shimadzu UV-2552 spectrophotometer (Tokyo, Japan). The results of prior research show that the OD_{600} is proportional to cell colony-forming-unit concentration (C , CFU/mL) for *P. aeruginosa* ($C = 1.65 \times 10^8 \times OD_{600}$, ref. [37]). Therefore, the ratio between OD_{600} for effluent samples and the influent was used to represent the ratio of cell concentration (C/C_0). The OD_{600} of the influent is 0.37, equivalent to a bacteria concentration C_0 of approximately 6×10^7 CFU/mL. All experiments were performed at room temperature of ~20°C.

Experiments were conducted for multiple cases, varying the status of the cells and/or the glass beads with respect to surface hydrophobicity. The column was newly packed if there was a change in the status of the cells or the glass beads. Multiple experiments were conducted for each cell/glass-beads combination, using the same packed column, varying the rhamnolipid concentration (0, 20, 40, 80 mg/L, and ionic strength control). Between each experiment, the column was flushed in series with AGW, hot NaOH solution (~100°C, 0.1 M, 1 h), and AGW to lyse and remove retained cells. After the OD_{600} of the effluent returned to 0, AGW injection continued for more than 30 PVs prior to the next injection of cell suspension. In addition, ionic strength control runs were implemented with cell suspension in AGW in the presence of additional 4.6 mg/L of NaCl (but no rhamnolipid), for which the mole concentration is equivalent to 40 mg/L rhamnolipid.

2.3 Data Analysis

A “clean-bed” colloid deposition model was used to delineate the transport processes [e.g., 38,39]. The deposition rate coefficient, k (h^{-1}), was determined using the equation:

$$k = -\frac{4Q}{\pi D^2 n L} \ln\left(\frac{C}{C_0}\right)$$

where Q (cm^3/h) is the flow rate, n is the porosity of the packed column, D (cm) is inner diameter of the column, and L (cm) is length of the column, and C/C_0 is the normalized

effluent concentration. The C/C_0 was determined for each experiment by averaging the C/C_0 value for the points at breakthrough curves between 1.8 and 2 pore volumes [18].

3 Results and Discussion

3.1 Effect of rhamnolipid on cell surface properties

Surface charge density and hydrophobicity of bacterial cells are considered to be two of the key factors affecting bacterial deposition and transport in porous media [7, 9]. The zeta potentials of glucose-grown cells and hexadecane-grown cells were close to each other ($-21 \sim -24$ mV). For both groups of cells, the zeta potentials did not change significantly with the increase of rhamnolipid concentration (Table 1), indicating that low concentrations of rhamnolipid do not have a strong effect on the cell surface charge of *P. aeruginosa*. This result is consistent with the results of our prior studies [33, 35]. The hexadecane-grown cells have more hydrophobic surfaces than glucose-grown cells (Table 1). The surface hydrophobicity of both groups of cells decreased with an increase of rhamnolipid concentration to 40 mg/L. This result is similar to the results of our prior experiments implemented under the same conditions [35], showing reproducibility.

3.2 Cell Transport in Saturated Glass Beads

Breakthrough curves (BTCs) for cell transport in glass beads are shown in Fig. 1. BTCs of PFBA are symmetrical and sharp for arrival and elution waves, indicating ideal transport and uniform packing of the column. BTCs of glucose-grown cells (more hydrophilic) in the absence of rhamnolipid show weak retention ($\sim 20\%$) and appear identical for both glass bead treatments, hydrophobic or hydrophilic surfaces (Fig. 1A and 1B). In contrast, retention of hexadecane-grown cells with higher surface hydrophobicity in the absence of rhamnolipid is much more significant than for the glucose-grown cells (Fig. 1C and 1D). Also, retention is slightly greater in the glass beads with hydrophobic surfaces ($\sim 85\%$) than with hydrophilic surfaces ($\sim 75\%$). For glass beads with either hydrophobic or hydrophilic surfaces, the obtained deposition rate coefficient, k , for hexadecane-grown cells is approximately three times greater than for glucose-grown cells (Fig. 2).

The results presented above indicate that the surface hydrophobicity of cells has a strong effect on transport of the cells in the packed columns. They also indicate that hydrophobic interaction plays a role in cell deposition, given that the highest k was obtained for the system comprising cells with higher surface hydrophobicity and glass beads with hydrophobic surfaces. This is consistent with the results of our prior study that Freundlich adsorption coefficients, K_f and $1/n$, were significantly larger for adsorption of *P. aeruginosa* ATCC 9027 cells with high surface hydrophobicity to glass beads with hydrophobic surfaces [35].

The presence of rhamnolipid enhances the retention of glucose-grown cells for both hydrophilic and hydrophobic glass beads (Fig. 1A and 1B). One possible reason for such enhancement is that the presence of rhamnolipid as an anionic surfactant increases the ionic strength of AGW (electrical conductivity of AGW increases with increasing rhamnolipid concentration, data not shown). Prior research has demonstrated that an increase of ionic

strength can inhibit bacterial transport [5, 24]. Indeed greater retention and a larger k are observed for the ionic strength control (ISC) experiment (Figs 1 A and 1B) in which additional NaCl (with mole concentration equivalent to 40 mg/L rhamnolipid) was added, compared to the standard experiment (no additional NaCl) with no rhamnolipid.

It is observed that the BTCs and magnitudes of retention for glucose cells with rhamnolipid C of 20 and 40 mg/L are similar to that for the ISC experiment for the hydrophobic glass beads (Fig. 1B). Conversely, the magnitude of retention for the experiment with rhamnolipid C of 80 mg/L is less than that for the ISC experiment. The magnitudes of retention for all rhamnolipid experiments conducted with the hydrophilic glass beads are less than the retention observed for the ISC experiment (Fig. 1A). These results suggest that rhamnolipid has some additional effect beyond that associated with increasing the ionic strength of the aqueous phase, and that such effect tends to facilitate cell transport. In addition, this effect appears to be of greater import for the systems with hydrophilic glass beads.

As shown in Fig. 1C and 1D, the presence of rhamnolipid at concentrations of 20 or 40 mg/L results in a decrease in retention of hexadecane-grown cells for both the hydrophilic and hydrophobic glass beads compared to the absence of rhamnolipid. This is in contrast to the results observed for the glucose-grown cells. The calculated deposition rate coefficient, k , decreases with increasing rhamnolipid concentration up to 40 mg/L (Fig. 2). Such a decrease in k is more significant for the glass beads with hydrophobic surfaces. An increase of rhamnolipid concentration to 80 mg/L, however, exhibits the inverse effect, with an increase in cell retention to a level comparable to that in the absence of rhamnolipid. These observations show that rhamnolipid at low concentrations can enhance the transport of cells with high surface hydrophobicity in the glass beads medium.

3.3 Pairing deposition rate coefficient with cell surface hydrophobicity

Comparing the results of cell transport and cell surface hydrophobicity, it is hypothesized that hydrophobic interaction may play an important role in cell retention. Furthermore, it is hypothesized that rhamnolipid affects retention by affecting cell surface hydrophobicity. To test this hypothesis, the relationship between the deposition rate coefficient (k) and cell surface hydrophobicity (presented as BATH rate) was examined. The results are presented in Fig. 3. It is observed that for all the data obtained (four combinations of cells and glass beads in the presence of rhamnolipid of different concentrations) there is a good linear relation between k and the cell BATH rates ($R^2=0.71$). Such linearity shows the importance of hydrophobic interaction in cell transport in porous media. Altering cell surface hydrophobicity, and in turn the hydrophobic interaction, is indicated to be an important mechanism for the rhamnolipid to affect cell transport in this study.

4 Conclusions

This study shows that transport of *P. aeruginosa* ATCC 9027 in glass beads depends on surface hydrophobicity of the cells and of the porous media. Monorhamnolipid at low concentrations, such as 40mg/L, reduced retention and enhanced the transport of hexadecane-grown cells with high surface hydrophobicity. The enhancement was more significant for the glass beads with hydrophobic surfaces than with hydrophilic surfaces. The

impact of rhamnolipid on cell surface hydrophobicity and thus the hydrophobic interaction between cells and glass beads, is hypothesized to play a role in the enhancement. The result of this study is of importance for improving rhamnolipid application in bioaugmentation processes for remediation of contaminated subsurface environments. Future research should focus on testing this role of rhamnolipid in naturally occurring porous media, in particular those with a large portion of high-hydrophobicity domains.

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Fig. 1. Breakthrough curves for transport of *P. aeruginosa* ATCC 9027 cells in glass beads. (A) glucose-grown cells (more hydrophilic surfaces), hydrophilic glass beads; (B) glucose-grown cells (more hydrophilic surfaces), hydrophobic glass beads; (C) hexadecane-grown cells (more hydrophobic surfaces), hydrophilic glass beads; (D) hexadecane-grown cells (more hydrophobic surfaces), hydrophobic glass beads. IS in legend refers to ionic strength.

Fig. 2.

Deposition rate coefficients for transport of *P. aeruginosa* ATCC 9027 cells in glass beads. GG cells, HG cells and GB in legend refer to glucose-grown cells, hexadecane-grown cells, and glass beads, respectively. IS refers to ionic strength.

Fig. 3.

Relation between deposition rate coefficients and cell BATH rate for *P. aeruginosa* ATCC 9027 transport in glass beads. GG cells, HG cells and GB in legend refer to glucose-grown cells, hexadecane-grown cells, and glass beads, respectively.

Table 1

Surface properties of *P. aeruginosa* ATCC 9027 cells in the presence of rhamnolipid.

Rhamnolipid concentration (mg/L)	Glucose-grown cells		Hexadecane-grown cells	
	Zeta potential (mV)	BATH rate	Zeta potential (mV)	BATH rate
0	-22.6 ± 0.4^a	0.297 ± 0.019	-20.9 ± 0.7	0.613 ± 0.022
20	-24.1 ± 0.3	0.224 ± 0.011	-22.0 ± 0.5	0.533 ± 0.006
40	-24.7 ± 0.6	0.131 ± 0.007	-24.3 ± 0.5	0.362 ± 0.060
80	-24.3 ± 0.6	0.253 ± 0.038	-23.6 ± 0.9	0.573 ± 0.033

^aMean $\pm$ Standard deviation of triplicate measurements